VEGF-D EXPRESSION IN BRAIN CANCER

CROSS REFERENCE TO RELATED APPLICATION

The present application claims the priority of U.S. provisional patent application number 60/268,089 filed February 12, 2001.

FIELD OF THE INVENTION

The invention relates to the fields of medicine, angiogenesis and neuro-oncology. More particularly, the invention relates to compositions and methods for detecting and treating malignant tumors.

BACKGROUND OF THE INVENTION

Cancer is presently the second leading cause of death in developed nations. Wingo et al., J. Reg. Management, 25:43-51 (1998). Despite recent research that has revealed many of the molecular mechanisms of tumorigenesis, few new treatments have achieved widespread clinical success in treating solid tumors. Current treatments for most malignancies thus remain gross resection, chemotherapy, and radiotherapy. While increasingly successful, each of these treatments still causes numerous undesired side effects. The primary cause of these side effects is that none of these conventional methods specifically targets only diseased cells. For example, surgery results in pain, traumatic injury to healthy tissue, and scarring. Radiotherapy and chemotherapy cause nausea, immune suppression, gastric ulceration and secondary tumorigenesis.

In an effort to develop techniques to more specifically target diseased cells, progress in tumor immunology has led to the discovery of antigens that are preferentially or specifically expressed by cancer cells. The identification of tumor-specific cellular markers has proven extremely valuable for diagnosing and assessing the progression of certain types of tumors. Antibodies specific for tumor cell markers or ligands that bind specifically to a tumor cell receptor have been successfully used in diagnostics, including both the characterization of excised tissue samples and *in vivo* imaging. Tumor-specific antibodies and ligands have also been used in the targeted delivery of cytotoxic molecules to specific tumor cells. Some tumor cell antigens are known to function in the pathogenesis of a cancer. Modulating the function of these antigens could impair the progression of the disease.

SUMMARY

The invention relates to the discovery that glioblastoma multiforme (GBM) strongly expresses vacular endothelial cell growth factor D (VEGF-D; also known as c-fos-induced growth factor or FIGF), an X-linked c-fos inducible angiogenic factor. By binding to and activating VEGF receptors 2 and 3 on endothelial cells, VEGF-D is thought to play an important role in causing the formation and development of new blood vessels that feed a cancerous tumor. Based on this discovery, central nervous system (CNS) cancers such as GBM can be diagnosed and treated using VEGF-D as a target tumor antigen. In addition, by disrupting the interaction between VEGF-D and its receptors, tumor angiogenesis can be inhibited.

Accordingly, the invention features a method for detecting a cancer in a brain tissue sample (e.g., one isolated from a human subject). This method includes the steps of providing the brain tissue sample; and analyzing the brain tissue sample for a VEGF-D marker such as a VEGF-D nucleic acid or VEGF-D protein. In this method, the step of analyzing the brain tissue sample can include comparing the quantity of expression of the VEGF-D marker to a first sample known to express detectable levels of the VEGF-D marker (a positive control) and a second sample known to not express detectable levels of the VEGF-D marker (a negative control).

VEGF-D nucleic acid expression can be analyzed by isolating RNA from the tissue sample, generating cDNAs from the isolated RNA, amplifying the cDNAs by PCR to generate a PCR product. Alternatively, VEGF-D nucleic acid expression can be analyzed by isolating nucleic acid from the tissue sample, and contacting the isolated nucleic acid with an oligonucleotide probe (e.g., a labeled oligonucleotide probe) that hybridizes under stringent hybridization conditions to the VEGF-D nucleic acid.

VEGF-D protein expression can be analyzed by contacting at least a portion of the brain tissue sample with a probe that specifically binds to the VEGF-D protein. The probe can be an antibody (e.g., a polyclonal or monoclonal antibody such as VD1), and can include a detectable label.

In another aspect, the invention features a method of modulating VEGF-D gene expression in a brain cancer cell. This method includes the steps of: providing a brain cancer cell that expresses a VEGF-D gene; and introducing into the cell an agent that modulates the expression of the VEGF-D gene in the cell. The agent can

be an oligonucleotide such as an antisense oligonucleotide that hybridizes under stringent hybridization conditions to a polynucleotide that encodes a VEGF-D protein.

The invention also features a method of identifying a test compound that modulates expression of a VEGF-D gene in a brain cancer cell (e.g., one derived from a human brain). This method includes the steps of: providing a brain cancer cell expressing a VEGF-D gene; contacting the cell with the test compound; and detecting a modulation in the expression of the VEGF-D gene. Detecting the modulation indicates that the test compound modulates expression of the VEGF-D gene. Modulation in the expression of the VEGF-D gene can be assessed by analyzing the cell for a change in the amount of a VEGF-D marker in the cell.

Also within the invention is a method for inhibiting angiogenesis associated with a brain cancer in a subject. This method includes the steps of: providing a molecule that interferes with VEGF-D binding to a VEGF-D receptor; and administering the molecule to the central nervous system of the subject in an amount effective to inhibit blood vessel development associated with the brain cancer. The molecule that interferes with VEGF-D binding to a VEGF-D receptor can be one that specifically binds VEGFR-2, VEGFR-3, or VEGF-D. This molecule can be an antibody such as a polyclonal or monoclonal antibody (e.g., VD1).

Unless otherwise defined, all technical terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Commonly understood definitions of molecular biology terms can be found in Rieger et al., Glossary of Genetics: Classical and Molecular, 5th edition, Springer-Verlag: New York, 1991; and Lewin, Genes V, Oxford University Press: New York, 1994.

By the term "cancer" is meant any disorder of cell growth that results in invasion and destruction of surrounding healthy tissue by abnormal cells.

As used herein, the term "gene" means a nucleic acid molecule that codes for a particular protein, or in certain cases, a functional or structural RNA molecule. For example, a VEGF-D gene encodes a VEGF-D protein. The phrase "nucleic acid" or a "nucleic acid molecule" means a chain of two or more nucleotides such as RNA (ribonucleic acid) and DNA (deoxyribonucleic acid). A "purified" nucleic acid molecule is one that is substantially separated from other nucleic acid sequences in a

cell or organism in which the nucleic acid naturally occurs (e.g., 30, 40, 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, 100% free of contaminants). The term includes, e.g., a recombinant nucleic acid molecule incorporated into a vector, a plasmid, a virus, or a genome of a prokaryote or eukaryote. Examples of purified nucleic acids include cDNAs, fragments of genomic nucleic acids, nucleic acids produced polymerase chain reaction (PCR), nucleic acids formed by restriction enzyme treatment of genomic nucleic acids, recombinant nucleic acids, and chemically synthesized nucleic acid molecules. A "recombinant" nucleic acid molecule is one made by an artificial combination of two otherwise separated segments of sequence, e.g., by chemical synthesis or by the manipulation of isolated segments of nucleic acids by genetic engineering techniques.

The phrases "VEGF-D gene," "VEGF-D polynucleotide," or "VEGF-D nucleic acid" as used herein mean a native VEGF-D-encoding nucleic acid sequence, e.g., the native human (Genbank Accession Nos. XM096295, NM004469 and Y12863), rat (Genbank Accession Nos. AY032728 and NM031761), mouse (Genbank Accession Nos. X99572 and D89628), and bovine (Genbank Accession No. AF099135) VEGF-D genes; a native form VEGF-D cDNA; a mature form of VEGF-D cDNA; a nucleic acid having sequences from which a VEGF-D cDNA can be transcribed; and/or allelic variants and homologs of the foregoing. The terms encompass double-stranded DNA, single-stranded DNA, and RNA.

As used herein, "protein" or "polypeptide" mean any peptide-linked chain of amino acids, regardless of length or post-translational modification, e.g., glycosylation or phosphorylation. A "purified" polypeptide is one that is substantially separated from other polypeptides in a cell or organism in which the polypeptide naturally occurs (e.g., 30, 40, 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, 100% free of contaminants).

By the phrase "VEGF-D protein" or "VEGF-D polypeptide" is meant an expression product of a VEGF-D gene such as a native VEGF-D protein, a mature form VEGF-D protein, or a protein that shares at least 65% (but preferably 75, 80, 85, 90 , 95, 96, 97 ,98, or 99%) amino acid sequence identity with one of the foregoing and displays a functional activity of a human native or mature VEGF-D protein. A "functional activity" of a protein is any activity associated with the physiological function of the protein. For example, functional activities of a native VEGF-D

protein may include VEGF receptor binding activity, selective expression in certain neoplastic tissues, and the ability to stimulate angiogenesis.

When referring to a nucleic acid molecule or polypeptide, the term "native" refers to a naturally-occurring (e.g., a "wild-type") nucleic acid or polypeptide. A "homolog" of a VEGF-D gene from one species of organism is a gene sequence encoding a VEGF-D polypeptide isolated from an organism of a different species. Similarly, a "homolog" of a native VEGF-D polypeptide is an expression product of a VEGF-D gene homolog.

As used herein, a "VEGF-D marker" is any molecule whose presence in a sample (e.g., a cell) indicates that a VEGF-D gene is expressed in the sample. VEGF-D markers include VEGF-D nucleic acids and VEGF-D proteins. "Expressing a VEGF-D gene" or like phrases mean that a sample contains a transcription product (e.g., messenger RNA, i.e., "mRNA") of a VEGF-D gene or a translation product of a VEGF-D protein-encoding nucleic acid (e.g., a VEGF-D protein). A cell expresses a VEGF-D gene when it contains a detectable level of a VEGF-D nucleic acid or a VEGF-D protein.

A "fragment" of a VEGF-D nucleic acid is a portion of a VEGF-D nucleic acid that is less than full-length and comprises at least a minimum length capable of hybridizing specifically with a native VEGF-D nucleic acid under stringent hybridization conditions. The length of such a fragment is preferably at least 15 nucleotides, more preferably at least 20 nucleotides, and most preferably at least 30 nucleotides of a native VEGF-D nucleic acid sequence. A "fragment" of a VEGF-D polypeptide is a portion of a VEGF-D polypeptide that is less than full-length (e.g., a polypeptide consisting of 5, 10, 15, 20, 30, 40, 50, 75, 100 or more amino acids of a native VEGF-D protein), and preferably retains at least one functional activity of a native VEGF-D protein

When referring to hybridization of one nucleic acid to another, "low stringency conditions" means in 10% formamide, 5X Denhart's solution, 6X SSPE, 0.2% SDS at 42°C, followed by washing in 1X SSPE, 0.2% SDS, at 50°C; "moderate stringency conditions" means in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.2X SSPE, 0.2% SDS, at 65°C; and "high stringency conditions" means in 50% formamide, 5X Denhart's solution, 5X SSPE, 0.2% SDS at 42°C, followed by washing in 0.1X SSPE, and 0.1% SDS at 65°C. The

phrase "stringent hybridization conditions" means low, moderate, or high stringency conditions

By the term "VEGF-D-specific antibody" is meant an antibody that binds a VEGF-D protein and displays no substantial binding to other naturally occurring proteins other than those sharing the same antigenic determinants as the VEGF-D protein. The term includes polyclonal and monoclonal antibodies as well as antibody fragments.

As used herein, "bind," "binds," or "interacts with" means that one molecule recognizes and adheres to a particular second molecule in a sample, but does not substantially recognize or adhere to other structurally unrelated molecules in the sample. Generally, a first molecule that "specifically binds" a second molecule has a binding affinity greater than about 10^5 to 10^6 moles/liter for that second molecule.

The term "labeled," with regard to a probe or antibody, is intended to encompass direct labeling of the probe or antibody by coupling (i.e., physically linking) a detectable substance to the probe or antibody.

Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions will control. The particular embodiments discussed below are illustrative only and not intended to be limiting.

DETAILED DESCRIPTION

The invention provides methods and compositions for diagnosing and treating malignant tumors including, in particular, brains cancers such as GBM. For example, according to the invention, brain cancer is diagnosed by analyzing a brain tissue sample for expression of a VEGF-D marker, such as a VEGF-D nucleic acid or a VEGF-D protein. Brain cancer is treated by introducing into the cells making up the cancer an agent that modulates VEGF-D gene expression in the cells. A brain cancer can also be treated by inhibiting the angiogenesis associated with the cancer by interfering with VEGF-D binding to a VEGF-D receptor, e.g., by administering a molecule that interferes with the VEGF-D/VEGFR-2 or -3 interaction to a subject suffering from a brain cancer. The invention also provides a method for identifying a

test compound that modulates expression of a VEGF-D gene in a brain cancer cell. To identify such a compound, a brain cancer cell expressing a VEGF-D gene is contacted with a test compound and analyzed for modulations in VEGF-D expression.

The below described preferred embodiments illustrate adaptations of these compositions and methods. Nonetheless, from the description of these embodiments, other aspects of the invention can be made and/or practiced based on the description provided below.

Biological Methods

Methods involving conventional molecular biology techniques are described herein. Such techniques are generally known in the art and are described in detail in methodology treatises such as Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989; and Current Protocols in Molecular Biology, ed. Ausubel et al., Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates). Various techniques using polymerase chain reaction (PCR) are described, e.g., in Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press: San Diego, 1990. PCR-primer pairs can be derived from known sequences by known techniques such as using computer programs intended for that purpose (e.g., Primer, Version 0.5, ©1991, Whitehead Institute for Biomedical Research, Cambridge, MA.). Methods for chemical synthesis of nucleic acids are discussed, for example, in Beaucage and Carruthers, Tetra. Letts. 22:1859-1862, 1981, and Matteucci et al., J. Am. Chem. Soc. 103:3185, 1981. Chemical synthesis of nucleic acids can be performed, for example, on commercial automated oligonucleotide synthesizers. Immunological methods (e.g., preparation of antigen-specific antibodies, immunoprecipitation, and immunoblotting) are described, e.g., in Current Protocols in Immunology, ed. Coligan et al., John Wiley & Sons, New York, 1991; and Methods of Immunological Analysis, ed. Masseyeff et al., John Wiley & Sons, New York, 1992. Conventional methods of gene transfer and gene therapy can also be adapted for use in the present invention. See, e.g., Gene Therapy: Principles and Applications, ed. T. Blackenstein, Springer Verlag, 1999; Gene Therapy Protocols (Methods in Molecular Medicine), ed. P.D. Robbins, Humana Press, 1997; and Retro-vectors for Human Gene Therapy, ed. C.P. Hodgson, Springer Verlag, 1996.

Method of Detecting a Cancer

The invention provides a method for detecting a cancer in a brain tissue sample by analyzing the brain tissue sample for a VEGF-D marker such as a VGEF-D nucleic acid or VEGF-D protein. A preferred version of this method includes comparing the quantity of VEGF-D marker expression in the brain tissue sample to one or more control samples. The control samples can be a positive control sample, i.e., a sample known to express detectable levels of the VEGF-D marker using the same method of analysis as used for the brain tissue sample; and a negative control sample, i.e., a sample known not to express detectable levels of the VEGF-D marker using the same method of analysis as used for the brain tissue sample. Use of positive and negative controls ensures accuracy of test results.

Cancerous Tumors

The invention is based on the discovery that brain cancer cells express higher levels of VEGF-D than do normal brain cells. In particular, as described below in more detail, GBM sections express high levels of VEGF-D compared to non-cancerous brain cells. Accordingly, preferred methods of the invention involve analyzing the brain cancer cells, particularly glioma and GBM cells, for VEGF-D expression. Various forms of glioma/GBM are described in more detail in Dai and Holland, Biochim. Biophys. Acta, 1551:M19-27, 2001 and Holland, Nat. Rev. Genetics, 2:120-129, 2001. In addition to brain cancers, the methods and compositions described herein might be used with other types of cancers that express high levels of VEGF-D.

Brain Tissue Samples

The invention provides methods for analyzing a brain tissue sample and administering a composition to a brain cancer in a mammal. Surgical techniques for obtaining brain tissue samples as well as administering various compositions to the brain are well known in the art. For example, such methods are described in standard neuro-surgery texts such as Atlas of Neurosurgery: Basic Approaches to Cranial and Vascular Procedures, by F. Meyer, Churchill Livingstone, 1999; Stereotactic and Image Directed Surgery of Brain Tumors, 1st ed., by David G.T. Thomas, WB Saunders Co., 1993; and Cranial Microsurgery: Approaches and Techniques, by L. N. Sekhar and E. De Oliveira, 1st ed., Thieme Medical Publishing, 1999. Methods for

obtaining and analyzing brain tissue are also described in Belay et al., Arch. Neurol. 58: 1673-1678 (2001); and Seijo et al., J. Clin. Microbiol. 38: 3892-3895 (2000).

Detection of VEGF-D Polynucleotides and Proteins

The invention encompasses methods for detecting the presence of a VEGF-D marker such as a VEGF-D protein or a VEGF-D nucleic acid in a biological sample as well as methods for measuring the level of a VEGF-D marker in a biological sample. Such methods are useful for diagnosing cancer associated with VEGF-D expression, e.g., brain cancer.

An exemplary method for detecting the presence or absence of a VEGF-D protein or nucleic acid in a biological sample involves obtaining a biological sample from a subject (e.g., a human patient), contacting the biological sample with a compound or an agent capable of detecting a VEGF-D protein or a nucleic acid encoding a VEGF-D protein (e.g., antibody, mRNA or genomic DNA), and analyzing binding of the compound or agent to the sample after washing. Those samples having specifically bound compound or agent express a VEGF-D protein or a nucleic acid encoding a VEGF-D protein.

A preferred agent for detecting a nucleic acid encoding a VEGF-D protein is a labeled nucleic acid probe capable of hybridizing to the nucleic acid encoding the VEGF-D protein. The nucleic acid probe can be, for example, all or a portion of a VEGF-D gene itself or all or a portion of a complement of a VEGF-D gene. Similarly, the probe can also be all or a portion of a VEGF-D gene variant, or all or a portion of a complement of a VEGF-D gene variant. For instance, oligonucleotides at least 15, 30, 50, 100, 250, or 500 nucleotides in length that specifically hybridize under stringent conditions to a native VEGF-D nucleic acid or a complement of a native VEGF-D nucleic acid can be used as probes within the invention. A preferred agent for detecting a VEGF-D protein is an antibody capable of binding to a VEGF-D protein, preferably an antibody with a detectable label. Such antibodies can be polyclonal, or more preferably, monoclonal. An intact antibody, or a fragment thereof (e.g., Fab or $F(ab')_2$) can be used.

Methods of the invention can be used to detect an mRNA encoding a VEGF-D protein, a genomic DNA encoding a VEGF-D protein, or a VEGF-D protein in a biological sample in vitro as well as in vivo. The quantity of expression of VEGF-D marker from a brain tissue sample may be compared with appropriate controls such as

a first sample known to express detectable levels of the VEGF-D marker (i.e., a positive control) and a second sample known to not express detectable levels of the VEGF-D marker (i.e., a negative control). For example, in vitro techniques for detection of mRNAs encoding a VEGF-D protein include PCR amplification methods, Northern hybridizations, and in situ hybridizations. In vitro techniques for detection of a VEGF-D protein include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations and immunofluorescence. In vitro techniques for detection of genomic DNA encoding VEGF-D include Southern hybridizations. Furthermore, in vivo techniques for detection of a VEGF-D protein include introducing a labeled anti-VEGF-D antibody into a biological sample or test subject. For example, the antibody can be labeled with a radioactive marker whose presence and location in a biological sample or test subject can be detected by standard imaging techniques.

Myriad detectable labels that may be used in a diagnostic assay for VEGF-D expression are known in the art. Nucleic acid probes, for example, may be labeled with chemiluminescent or radioactive substances. The amount of labeled probe bound to a VEGF-D marker may then be assessed using photographic or X-ray film or other suitable methods for detecting luminescence or radioactivity. Antibodies used in methods for detecting VEGF-D protein may be conjugated to a detectable label, e.g., an enzyme such as horseradish peroxidase. Antibodies labeled with horseradish peroxidase can be detected by adding an appropriate substrate that produces a color change in the presence of horseradish peroxidase. Several other detectable labels that may be used are known. Common examples of these include alkaline phosphatase, fluorescent compounds, luminescent compounds, colloidal gold, magnetic particles, biotin, radioisotopes, and enzymes.

Nucleic Acids Encoding VEGF-D Proteins

Methods of the present invention relate to VEGF-D nucleic acids. Preferred nucleic acid molecules for use in the invention include native human (Genbank Accession Nos. XM096295, NM004469 and Y12863), rat (AY032728, NM031761), mouse (X99572, D89628), and bovine (AF099135) VEGF-D polynucleotides. Nucleic acid molecules utilized in the present invention may be in the form of RNA or in the form of DNA (e.g., cDNA, genomic DNA, and synthetic DNA). The DNA may be double-stranded or single-stranded, and if single-stranded may be the coding

(sense) strand or non-coding (anti-sense) strand. In addition to a coding sequence which encodes a native VEGF-D protein, other nucleic acid molecules that can be used in the invention include variants of a native VEGF-D gene such as those that encode fragments, analogs and derivatives of a native VEGF-D protein. Such variants may be, e.g., a naturally occurring allelic variant of a native VEGF-D gene or a homolog of a native VEGF-D gene.

Probes and Primers

Nucleic acids that hybridize under stringent conditions to VEGF-D nucleic acid or the complement of VEGF-D nucleic acid can be used in the invention. For example, such nucleic acids can be those that hybridize to VEGF-D nucleic acid or the complement of a VEGF-D nucleic acid under low stringency conditions, moderate stringency conditions, or high stringency conditions. Preferred such nucleic acids are those having a nucleotide sequence that is the complement of all or a portion of VEGF-D nucleic acid. Others that might be used include polynucleotides that share at least 65% (e.g., 65, 70, 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, and 99%) sequence identity to a native VEGF-D nucleic acid or the complement of VEGF-D nucleic acid. Nucleic acids that hybridize under stringent conditions to or share at least 65% sequence identity with VEGF-D nucleic acid or the complement of VEGF-D nucleic acid can be obtained by techniques known in the art such as by making mutations in a native VEGF-D gene, or by isolation from an organism expressing such a nucleic acid (e.g., an allelic variant).

Methods of the invention utilize oligonucleotide probes (i.e., isolated nucleic acid molecules conjugated with a detectable label or reporter molecule, e.g., a radioactive isotope, ligand, chemiluminescent agent, or enzyme); and oligonucleotide primers (i.e., isolated nucleic acid molecules that can be annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, e.g., a DNA polymerase). Primer pairs can be used for amplification of a nucleic acid sequence, e.g., by the polymerase chain reaction (PCR) or other conventional nucleic-acid amplification methods.

PCR primers can be used to amplify VEGF-D nucleic acids using known PCR and RT-PCR protocols. Such primers can be designed according to known methods

as PCR primer design is generally known in the art. See, e.g., methodology treatises such as Basic Methods in Molecular Biology, 2nd ed., ed. Davis et al., Appleton & Lange, Norwalk, CN, 1994; and Molecular Cloning: A Laboratory Manual, 2nd ed., vol.1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989. PCR primer pairs previously used to amplify a VEGF-D nucleic acid sequence are described in O-charoenrat et al., Cancer 92:556-568 (2001); George et al., Neoplasia 3:420-427 (2001); and Kurebayashi et al., Jpn. J. Cancer Res. 90:977-981 (1999).

Probes and primers utilized in methods of the invention are generally 15 nucleotides or more in length, preferably 20 nucleotides or more, more preferably 25 nucleotides, and most preferably 30 nucleotides or more. Preferred probes and primers are those that hybridize to a native VEGF-D gene (or cDNA or mRNA) sequence under high stringency conditions, and those that hybridize to VEGF-D gene homologs under at least moderately stringent conditions. Preferably, probes and primers according to the present invention have complete sequence identity with a native VEGF-D nucleic acid sequence. However, probes differing from this sequence that retain the ability to hybridize to a native VEGF-D gene sequence under stringent conditions may be designed by conventional methods and used in the invention. Primers and probes based on the VEGF-D gene sequences disclosed herein can be used to confirm (and, if necessary, to correct) the disclosed VEGF-D gene sequences by conventional methods, e.g., by re-cloning and sequencing a native VEGF-D gene or cDNA.

VEGF-D Proteins

The invention also provides methods involving VEGF-D proteins. VEGF-D is initially synthesized as a precursor protein containing N-terminal and C-terminal propeptides in addition to the VEGF-D homology domain (VHD). Achen et al., PNAS 95:548-553 (1998). During biosynthesis, these propeptides are proteolytically cleaved from the VHD, generating a mature, secreted form consisting of noncovalent dimers of the VHD. Stacker et al., J. Biol. Chem. 274:32127-32136 (1999). The mature form binds both VEGFR-2 and VEGFR-3 with much higher affinity than does the unprocessed form of VEGF-D.

Methods of the present invention may utilize a purified VEGF-D protein. A preferred form of VEGF-D is a purified native human VEGF-D protein that has the

amino acid sequence deposited with SwissProt as accession No. 043915. Other forms of VEGF-D include those of mouse (SwissProt accession No. P97946), rat (SwissProt accession No. 035251), and bovine (SwissProt accession No. Q9GCX1).

Variants of native VEGF-D proteins such as fragments, analogs and derivatives of native VEGF-D proteins may also be used in methods of the invention. Such variants include, e.g., a polypeptide encoded by a naturally occurring allelic variant of a native VEGF-D gene, a polypeptide encoded by an alternative splice form of a native VEGF-D gene, a polypeptide encoded by a homolog of a native VEGF-D gene, and a polypeptide encoded by a non-naturally occurring variant of a native VEGF-D gene.

VEGF-D protein variants have a peptide sequence that differs from a native VEGF-D protein in one or more amino acids. The peptide sequence of such variants can feature a deletion, addition, or substitution of one or more amino acids of a native VEGF-D polypeptide. Amino acid insertions are preferably of about 1 to 4 contiguous amino acids, and deletions are preferably of about 1 to 10 contiguous amino acids. In some applications, variant VEGF-D proteins substantially maintain a native VEGF-D protein functional activity (e.g., association with cancer or ability to modulate angiogenesis). For other applications, variant VEGF-D proteins lack or feature a significant reduction in a VEGF-D protein functional activity. Where it is desired to retain a functional activity of native VEGF-D protein, preferred VEGF-D protein variants can be made by expressing nucleic acid molecules within the invention that feature silent or conservative changes. Variant VEGF-D proteins with substantial changes in functional activity can be made by expressing nucleic acid molecules within the invention that feature less than conservative changes.

VEGF-D protein fragments corresponding to one or more particular motifs and/or domains or to arbitrary sizes, for example, at least 5, 10, 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, and 350 amino acids in length may be utilized in methods of the present invention. Isolated peptidyl portions of VEGF-D proteins can be obtained by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, a VEGF-D protein used in methods of the present invention may be arbitrarily divided into fragments of

desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments which can function as either agonists or antagonists of a native VEGF-D protein.

Methods of the invention may also involve recombinant forms of the VEGF-D proteins. Recombinant polypeptides preferred by the present invention, in addition to native VEGF-D protein, are encoded by a nucleic acid that has at least 85% sequence identity (e.g., 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100%) with a native VEGF-D nucleic acid sequence. In a preferred embodiment, variant VEGF-D proteins lack one or more functional activities of native VEGF-D protein (e.g., activating VEGF receptors).

VEGF-D protein variants can be generated through various techniques known in the art. For example, VEGF-D protein variants can be made by mutagenesis, such as by introducing discrete point mutation(s), or by truncation. Mutation can give rise to a VEGF-D protein variant having substantially the same, or merely a subset of the functional activity of a native VEGF-D protein. Alternatively, antagonistic forms of the protein can be generated which are able to inhibit the function of the naturally occurring form of the protein, such as by competitively binding to another molecule that interacts with VEGF-D protein. In addition, agonistic forms of the protein may be generated that constitutively express one or more VEGF-D functional activities. Other variants of VEGF-D proteins that can be generated include those that are resistant to proteolytic cleavage, as for example, due to mutations that alter protease target sequences. Whether a change in the amino acid sequence of a peptide results in a VEGF-D protein variant having one or more functional activities of a native VEGF-D protein can be readily determined by testing the variant for a native VEGF-D protein functional activity.

Nucleic acid molecules encoding VEGF-D fusion proteins may be used in methods of the invention. Such nucleic acids can be made by preparing a construct (e.g., an expression vector) that expresses a VEGF-D fusion protein when introduced into a suitable host. For example, such a construct can be made by ligating a first polynucleotide encoding a VEGF-D protein fused in frame with a second

polynucleotide encoding another protein such that expression of the construct in a suitable expression system yields a fusion protein.

As another example, VEGF-D protein variants can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential VEGF-D protein sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) Tetrahedron 39:3; Itakura et al. (1981) Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules, ed. AG Walton, Amsterdam: Elsevier pp 273-289; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) Science 249:386-390; Roberts et al. (1992) Proc. Natl. Acad. Sci. USA 89:2429-2433; Devlin et al. (1990) Science 249: 404-406; Cwirla et al. (1990) Proc. Natl. Acad. Sci. USA 87: 6378-6382; as well as U.S. Pat. Nos. 5,223,409; 5,198,346; and 5,096,815).

Similarly, a library of coding sequence fragments can be provided for a VEGF-D gene clone in order to generate a variegated population of VEGF-D protein fragments for screening and subsequent selection of fragments having one or more native VEGF-D protein functional activities. A variety of techniques are known in the art for generating such libraries, including chemical synthesis. In one embodiment, a library of coding sequence fragments can be generated by (i) treating a double-stranded PCR fragment of a VEGF-D gene coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule; (ii) denaturing the double-stranded DNA; (iii) renaturing the DNA to form double-stranded DNA which can include sense/antisense pairs from different nicked products; (iv) removing single-stranded portions from reformed duplexes by treatment with S1 nuclease; and (v) ligating the resulting fragment library into an expression vector. By this exemplary method, an expression library can be derived which codes for N-terminal, C-terminal and internal fragments of various sizes.

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations or truncation, and for screening

cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of VEGF-D gene variants. The most widely used techniques for screening large gene libraries typically involve cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected.

Combinatorial mutagenesis has a potential to generate very large libraries of mutant proteins, e.g., in the order of 10^{26} molecules. To screen a large number of protein mutants, techniques that allow one to avoid the very high proportion of non-functional proteins in a random library and simply enhance the frequency of functional proteins (thus decreasing the complexity required to achieve a useful sampling of sequence space) can be used. For example, recursive ensemble mutagenesis (REM), an algorithm that enhances the frequency of functional mutants in a library when an appropriate selection or screening method is employed, might be used. Arkin and Yourvan (1992) Proc. Natl. Acad. Sci. USA 89:7811-7815; Yourvan et al. (1992) Parallel Problem Solving from Nature, 2., In Maenner and Manderick, eds., Elsevier Publishing Co., Amsterdam, pp. 401-410; Delgrave et al. (1993) Protein Engineering 6(3):327-331.

Methods of the invention may utilize mimetics, e.g. peptide or non-peptide agents, that are able to disrupt binding of a VEGF-D protein to other proteins or molecules with which a native VEGF-D protein interacts. Thus, the mutagenic techniques described herein can also be used to map which determinants of VEGF-D protein participate in the intermolecular interactions involved in, for example, binding of a VEGF-D protein to other proteins which may function upstream (e.g., activators or repressors of VEGF-D functional activity) of the VEGF-D protein or to proteins or nucleic acids which may function downstream of the VEGF-D protein (e.g. VEGF receptors 2 and 3), and whether such molecules are positively or negatively regulated by the VEGF-D protein. To illustrate, the critical residues of a VEGF-D protein which are involved in molecular recognition of, for example, the VEGF-D protein or other components upstream or downstream of the VEGF-D protein can be determined and used to generate VEGF-D protein-derived peptidomimetics which competitively

inhibit binding of the VEGF-D protein to that moiety. By employing scanning mutagenesis to map the amino acid residues of a VEGF-D protein that are involved in binding other proteins (e.g., transmembrane receptor), peptidomimetic compounds can be generated which mimic those residues of a native VEGF-D protein. Such mimetics may then be used to interfere with the normal function of a VEGF-D protein. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al. in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al. in Peptides: Chemistry and Biology, G. R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopepitides (Ewenson et al. (1986) J. Med. Chem. 29:295; and Ewenson et al. in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, Ill, 1985), eta-turn dipeptide cores (Nagai et al. (1985) Tetrahedron Lett 26:647; and Sato et al. (1986) J. Chem. Soc. Perkin. Trans. 1:1231), and beta-aminoalcohols (Gordon et al. (1985) Biochem. Biophys. Res. Commun. 126:419; and Dann et al. (1986) Biochem. Biophys. Res. Commun. 134:71). VEGF-D proteins may also be chemically modified to create VEGF-D protein derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of VEGF-D protein can be prepared by linking the chemical moieties to functional groups on amino acid side chains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

Antibodies

Antibodies that specifically bind VEGF-D proteins or VEGF-D recptors can be used in methods of the invention, for example, in the detection of VEGF-D protein markers or to interfere with VEGF-D/VEGFR-2 and VEGF-D/VEGFR-3 interactions. Antibodies used in methods of the invention include polyclonal antibodies and, in addition, monoclonal antibodies, single chain antibodies, Fab fragments, $F(ab')_2$ fragments, and molecules produced using a Fab expression library. Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, can be prepared using the VEGF-D proteins described above and standard hybridoma

technology (see, for example, Kohler et al., Nature 256:495, 1975; Kohler et al., Eur. J. Immunol. 6:511, 1976; Kohler et al., Eur. J. Immunol. 6:292, 1976; Hammerling et al., "Monoclonal Antibodies and T Cell Hybridomas," Elsevier, N.Y., 1981; Ausubel et al., supra). In particular, monoclonal antibodies can be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture such as described in Kohler et al., Nature 256:495, 1975, and U.S. Pat. No. 4,376,110; the human B-cell hybridoma technique (Kosbor et al., Immunology Today 4:72, 1983; Cole et al., Proc. Natl. Acad. Sci. USA 80:2026, 1983), and the EBV-hybridoma technique (Cole et al., "Monoclonal Antibodies and Cancer Therapy," Alan R. Liss, Inc., pp. 77-96, 1983). Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof.

Antibodies that specifically recognize and bind to VEGF-D are useful in methods of the present invention. For example, such antibodies can be used in an immunoassay to monitor the level of a VEGF-D protein produced by a mammal (e.g., to determine the amount or subcellular location of a VEGF-D protein). Methods of the invention may also utilize antibodies, for example, in the detection of a VEGF-D protein in a biological sample. Antibodies also can be used in a screening assay to measure the effect of a candidate compound on expression or localization of a VEGF-D protein. Additionally, such antibodies can be used to interfere with the interaction of a VEGF-D protein and other molecules that bind the VEGF-D protein (e.g., VEGF receptors 2, 3 and VEGF-D). In a similar manner, antibodies that specifically bind VEGF receptors 2, 3 can be used to interfere with this interaction.

Particularly useful antibodies include monoclonal antibodies raised against the bioactive VHD of human VEGF-D. The production of VD1, a monoclonal antibody against the VHD of human VEGF-D raised in mice is described in Achen et al., Eur. J. Biochem. 267:2505-2515. VD1 binds both unprocessed and fully processed VEGF-D and is able to block the interaction of VEGF-D with both VEGFR-2 and VEGFR-3. VD1 is a preferred antibody for the immunohistochemical detection of bioactive VEGF-D.

Modulating VEGF-D Expression

Antisense, Ribozyme, Triplex Techniques

Another aspect of the invention relates to the use of purified antisense nucleic acids to inhibit expression of VEGF-D. Antisense nucleic acid molecules within the

invention are those that specifically hybridize (e.g. bind) under cellular conditions to cellular mRNA and/or genomic DNA encoding a VEGF-D protein in a manner that inhibits expression of the VEGF-D protein, e.g., by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix.

Antisense constructs can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes a VEGF-D protein. Alternatively, the antisense construct can take the form of an oligonucleotide probe generated ex vivo which, when introduced into a VEGF-D protein expressing cell, causes inhibition of VEGF-D protein expression by hybridizing with an mRNA and/or genomic sequences coding for VEGF-D protein. Such oligonucleotide probes are preferably modified oligonucleotides that are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and are therefore stable in vivo. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see, e.g., U.S. Pat. Nos. 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol et al. (1988) Biotechniques 6:958-976; and Stein et al. (1988) Cancer Res 48:2659-2668. With respect to antisense DNA, oligodeoxyribonucleotides derived from the translation initiation site, e.g., between the -10 and +10 regions of a VEGF-D protein encoding nucleotide sequence, are preferred.

Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to VEGF-D mRNA. The antisense oligonucleotides will bind to VEGF-D mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex or triplex. One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex. Oligonucleotides that are complementary to the 5' end of the message, e.g., the 5' untranslated sequence up to

and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have been shown to be effective at inhibiting translation of mRNAs as well. (Wagner, R. (1994) Nature 372:333). Therefore, oligonucleotides complementary to either the 5' or 3' untranslated, non-coding regions of a VEGF-D gene could be used in an antisense approach to inhibit translation of endogenous VEGF-D mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should preferably include the complement of the AUG start codon. Although antisense oligonucleotides complementary to mRNA coding regions are generally less efficient inhibitors of translation, these could still be used in the invention. Whether designed to hybridize to the 5', 3' or coding region of a VEGF-D mRNA, preferred antisense nucleic acids are less that about 100 (e.g., less than about 30, 25, 20, or 18) nucleotides in length. Generally, in order to be effective, the antisense oligonucleotide should be 18 or more nucleotides in length.

Specific antisense oligonucleotides can be tested for effectiveness using in vitro studies to assess the ability of the antisense oligonucleotide to inhibit gene expression. Preferably such studies (1) utilize controls (e.g., a non-antisense oligonucleotide of the same size as the antisense oligonucleotide) to distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides, and (2) compare levels of the target RNA or protein with that of an internal control RNA or protein.

Antisense oligonucleotides of the invention may include at least one modified base or sugar moiety. Exemplary modified bases include 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xanthine, 4-acetylcytosine, 5-(carboxyhydroxyethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouricil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-idimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic

acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine. Exemplary modified sugar moieties include arabinose, 2-fluoroarabinose, xylulose, and hexose. The antisense oligonucleotides of the invention may in some embodiments include at least one modified phosphate backbone such as a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, or a formacetal or analog thereof.

Antisense oligonucleotides within the invention might also be an alpha-anomeric oligonucleotide. See, Gautier et al. (1987) Nucl. Acids Res. 15:6625-6641. For example, the antisense oligonucleotide can be a 2'-0-methylribonucleotide (Inoue et al. (1987) Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al. (1987) FEBS Lett. 215:327-330). Oligonucleotides of the invention may be synthesized by standard methods known in the art, e.g by use of an automated DNA synthesizer. Phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988) Nucl. Acids Res. 16:3209). Methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al. (1988) Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451).

Methods of the invention also utilize techniques for delivering one or more of the above-described nucleic acid molecules into cells that express VEGF-D. A number of methods have been developed for delivering antisense DNA or RNA into cells. For instance, antisense molecules can be introduced directly into a cell by electroporation, liposome-mediated transfection, CaCl-mediated transfection, viral vector infection, or using a gene gun. Modified nucleic acid molecules designed to target the desired cells (e.g., antisense oligonucleotides linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be used. To achieve high intracellular concentrations of antisense oligonucleotides (as may be required to suppress translation on endogenous mRNAs), a preferred approach utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong promoter (e.g., the CMV promoter).

Ribozyme molecules designed to catalytically cleave VEGF-D mRNA transcripts can also be used to prevent translation of VEGF-D mRNAs and expression of VEGF-D proteins (See, e.g., Wright and Kearney, Cancer Invest. 19:495, 2001;

Lewin and Hauswirth, Trends Mol. Med. 7:221, 2001; Sarver et al. (1990) Science 247:1222-1225 and U.S. Pat. No. 5,093,246). As one example, hammerhead ribozymes that cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA might be used so long as the target mRNA has the following common sequence: 5'-UG-3'. See, e.g., Haseloff and Gerlach (1988) Nature 334:585-591. To increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts, a ribozyme should be engineered so that the cleavage recognition site is located near the 5' end of the target VEGF-D mRNA. Ribozymes within the invention can be delivered to a cell using a vector as described below.

Other methods can also be used to reduce VEGF-D gene expression in a cell. For example, VEGF-D gene expression can be reduced by inactivating or "knocking out" the VEGF-D gene or its promoter using targeted homologous recombination. See, e.g, Kempin et al., Nature 389: 802 (1997); Smithies et al. (1985) Nature 317:230-234; Thomas and Capecchi (1987) Cell 51:503-512; and Thompson et al. (1989) Cell 5:313-321. For instance, a mutant, non-functional VEGF-D gene variant (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous VEGF-D gene (either the coding regions or regulatory regions of the VEGF-D gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express VEGF-D protein in vivo.

VEGF-D gene expression might also be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the VEGF-D gene (i.e., the VEGF-D promoter and/or enhancers) to form triple helical structures that prevent transcription of the VEGF-D gene in target cells. See generally, Helene, C. (1991) Anticancer Drug Des. 6(6):569-84; Helene, C., et al. (1992) Ann. N.Y. Acad. Sci. 660:27-36; and Maher, L. J. (1992) Bioassays 14(12):807-15. Nucleic acid molecules to be used in this technique are preferably single stranded and composed of deoxyribonucleotides. The base composition of these oligonucleotides should be selected to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizable stretches of either purines or pyrimidines to be present on one strand of a duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC triplets across the three associated strands of the resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-

rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in CGC triplets across the three strands in the triplex. The potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizable stretch of either purines or pyrimidines to be present on one strand of a duplex.

The antisense RNA and DNA, ribozyme, and triple helix molecules that can be used with methods of the invention may be prepared by any method known in the art for the synthesis of DNA and RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides and oligoribonucleotides well known in the art such as for example solid phase phosphoramide chemical synthesis. RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

Modulating Fra-1 Expression and Binding

Other methods that can be used to reduce VEGF-D expression include modulating the expression and activity of Fra-1. The gene for VEGF-D harbors an optimal AP-1 binding site in its promoter region and is activated by Fra-1, an AP-1 transcription factor. Accordingly, one may modulate the activity or expression of this upstream activator in a method for reducing VEGF-D expression. Fra-1 gene expression can be modulated by one of the above-described methods, e.g., antisense, ribozyme, or triple helical methods.

Methods of the invention may also involve targeting the interaction of Fra-1 with its binding partners in an effort to block activation of the VEGF-D promoter by Fra-1. Fra-1 cannot activate gene expression itself, as it requires heterodimerization

with Jun proteins to do so. c-Jun and JunB in particular are preferable partners for Fra-1 and, in the process of Fra-1 upregulation in response to Ras activation, c-Jun is primarily utilized as the binding partner with Fra-1. Therefore, nucleic acids which encode proteins that bind Fra-1 and preclude binding of Fra-1 to c-Jun may be used to block activation of the VEGF-D promoter by Fra-1. Methods of the invention may alternatively utilize mimetics, e.g. peptide or non-peptide agents, that are able to disrupt binding of a Fra-1 protein to other proteins or molecules (e.g., c-Jun) with which the native Fra-1 protein interacts. Alternatively, antagonistic forms of the Fra-1 protein can be generated which are able to inhibit the function of the naturally occurring form of the protein, such as by competitively binding to another molecule that interacts with Fra-1 protein. In particular, a variant form of Fra-1 that is able to heterodimerize with c-Jun yet is incapable of binding the VEGF-D promoter is suitable for use in a method to inhibit VEGF-D gene expression. Fra-1 protein variants can be generated through various techniques known in the art. For example, Fra-1 protein variants can be made by mutagenesis, such as by introducing an insertion, deletion or a discrete point mutation(s).

Gene Therapy

Methods of the present invention include the delivery of nucleic acids and proteins into a mammalian subject for inhibiting angiogenesis or otherwise treating a cancer. A number of gene therapy technologies may be employed to this end. Methods and compositions involving gene therapy vectors are generally known in the art and are described in methodology references such as Viral Vectors, eds. Yakov Gluzman and Stephen H. Hughes, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1988; Retroviruses, Cold Spring Harbor Laboratory Press, Plainview, NY, 2000; Gene Therapy Protocols (Methods in Molecular Medicine), ed. Jeffrey R. Morgan, Humana Press, Totawa, NJ, 2001; and Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989.

Various methods for inhibiting angiogenesis utilize anti-angiogenic agents that may be administered to a mammalian subject, including a human, by any suitable technique. Techniques using viral vectors for the introduction of nucleic acids encoding VEGF-D mutants, Fra-1 binding species, antisense RNAs and VEGF receptor antagonists into cells may be utilized in methods of the invention. Preferred

viral vectors exhibit low toxicity to the host cell and produce therapeutic quantities of anti-angiogenic molecules in a tissue-specific manner. Viral vector methods and protocols are reviewed in Kay et al. Nature Medicine 7:33-40, 2001.

Methods for use of recombinant adenoviruses as gene therapy vectors are discussed, for example, in W.C. Russell, Journal of General Virology 81:2573-2604, 2000, and Bramson et al., Curr. Opin. Biotechnol. 6:590-595, 1995. Viral vectors utilized in methods of the invention can also include Adeno-Associated Virus (AAV) vectors. Methods for use of recombinant AAV vectors are discussed, for example, in Tal, J., J. Biomed. Sci. 7:279-291, 2000 and Monahan and Samulski, Gene Therapy 7:24-30, 2000. Methods for use of Herpes Simplex Virus (HSV) vectors are discussed, for example, in Cotter and Robertson, Curr. Opin. Mol. Ther. 1:633-644, 1999. A preferred HSV vector is engineered from HSV type I, is deleted of the immediate early genes (IE) and contains a tissue-specific (*e.g.*, brain) promoter operably linked to a nucleic acid encoding a VEGF-D mutant, Fra-1 binding species, antisense RNA, or VEGF receptor antagonist. HSV amplicon vectors may also be used in methods of the invention. Viral vectors utilized in methods of the present invention may also include replication-defective lentiviral vectors, including HIV. Methods for use of lentiviral vectors are discussed, for example, in Vigna and Naldini, J. Gene Med. 5:308-316, 2000 and Miyoshi et al., J. Virol. 72:8150-8157, 1998.

To combine advantageous properties of two viral vector systems, hybrid viral vectors may be used to deliver a nucleic acid encoding a VEGF-D mutant, Fra-1 binding species, antisense RNA, or VEGF receptor antagonist to a target tissue (*e.g.*, brain). Standard techniques for the construction of hybrid vectors are well-known to those skilled in the art. Such techniques can be found, for example, in Sambrook, et al., In Molecular Cloning: A laboratory manual. Cold Spring Harbor, NY or any number of laboratory manuals that discuss recombinant DNA technology. Adenovirus/AAV hybrid vectors are discussed in Lieber et al., J. Virol. 73:9314-9324, 1999.

Retroviral/Adenovirus hybrid vectors are discussed in Zheng et al., Nature Biotechnol. 18:176-186, 2000. Retroviral genomes contained within an Adenovirus may integrate within the host cell genome and effect stable gene expression of a VEGF-D mutant, Fra-1 binding species, antisense RNA, or VEGF receptor

antagonist. More than one promoter can be present in a vector. Accordingly, more than one heterologous gene can be expressed by a vector. Further, the vector can comprise a sequence which encodes a signal peptide or other moiety which facilitates the secretion of a VEGF-D mutant or VEGF receptor antagonist product, for example, from the host cell.

For use in methods of the invention, other nucleotide sequence elements which facilitate expression of a VEGF-D mutant, Fra-1 binding species, antisense RNA, or VEGF receptor antagonist gene and cloning of the vector are further contemplated. The presence of enhancers upstream of the promoter or terminators downstream of the coding region, for example, can facilitate expression. Within vectors used in methods of the invention, the presence of elements which enhance brain-specific expression of anti-angiogenic molecules may be useful for gene therapy.

Several non-viral methods for introducing a VEGF-D mutant, Fra-1 binding species, antisense RNA, or VEGF receptor antagonist gene into host cells are also within the scope of the invention. For a review of non-viral methods, see Nishikawa and Huang, Human Gene Ther. 12:861-870, 2001. Various techniques employing plasmid DNA for the introduction of a VEGF-D mutant, Fra-1 binding species, antisense RNA, or VEGF receptor antagonist gene into tissue are provided for according to the invention. Such techniques are generally known in the art and are described in references such as Ilan, Y., Curr. Opin. Mol. Ther. 1:116-120, 1999, Wolff, J.A., Neuromuscular Disord. 7:314-318, 1997 and Arztl, Z., Fortbild Qualitatssich 92:681-683, 1998.

Protein transduction offers an alternative to gene therapy for the delivery of therapeutic proteins into target cells, and techniques of protein transduction may be used within methods of the invention. Protein transduction is the internalization of proteins into a host cell, from the external environment. The internalization process relies on a protein or peptide which is able to penetrate the cell membrane. The transducing property of such a protein or peptide can be conferred upon proteins (Fra-1 binding species, for example) which are expressed as fusion proteins with them. Commonly used protein transduction vehicles include the antennapedia peptide, the HIV TAT protein transduction domain and the herpes simplex virus VP22 protein. Such vehicles are reviewed in Ford et al., Gene Ther. 8:1-4, 2001.

Method for Identifying a Test Compound

That Modulates VEGF-D Gene Expression

The invention provides for a method of identifying a test compound that modulates expression of a VEGF-D gene in a brain cancer cell. One such method involves providing a cell that expresses VEGF-D and at least one test compound, contacting the cell with the test compound, and detecting whether or not the test compound modulates VEGF-D expression. Those compounds resulting specifically in altered levels (increased or decreased levels) of VEGF-D protein are those that specifically modulate VEGF-D expression. For example, a library of molecules can be screened by providing brain cancer cells expressing VEGF-D and contacting the cells with the library and examining the cells for changes in VEGF-D expression. Changes in VEGF-D expression may be assessed by analyzing changes in VEGF-D marker (e.g. VEGF-D protein and VEGF-D mRNA) levels.

Interfering with VEGF-D/VEGFR Interactions

VEGF-D Protein Specific Antibodies and Binding Molecules

VEGF-D binding molecules according to the invention include cell surface receptor tyrosine kinases VEGFR-2 and VEGFR-3. VEGFR-2, also known as Flk1 or KDR, is specifically expressed on endothelial cells during embryonic and tumor development. Plate et al., Cancer Research 53: 5822-5827 (1993). VEGFR-3, also known as Flt4, is expressed on venous endothelial cells during early embryogenesis but subsequently becomes restricted to the endothelial cells of lymphatic vessels. Kaipainen et al., PNAS 92: 3566-3570 (1995). VEGF-D binds to activates both VEGFR-2 and VEGFR-3. Agents that interfere with VEGF-D binding to a VEGF receptor include antibodies such as those that specifically bind to VEGF-D, VEGFR-2, and VEGFR-3. Those that block binding between VEGF-D and one of its receptors are preferred. Particularly useful antibodies include monoclonal antibodies raised against the bioactive VHD of human VEGF-D. VD1, a monoclonal antibody against the VHD of human VEGF-D, binds both unprocessed and fully processed VEGF-D and is able to block the interaction of VEGF-D with both VEGFR-2 and VEGFR-3. VD1 is a preferred antibody for neutralization (e.g., blocking the VEGF-D/VEGFR interaction) of bioactive VEGF-D. Such an agent can be used, according to the invention, to block the mitogenic response of human microvascular endothelial cells to VEGF-D.

Receptor Antagonists - VEGF-D/VEGFR Binding Mutants

Other binding molecules may be used to interfere with VEGF-D binding to a VEGF receptor. Nucleic acids encoding binding mutants as well as binding mutant proteins may be used in methods of the invention. Such molecules include variant VEGF-D proteins that contain a VHD yet lack receptor binding activity. An example of such a variant is a dominant negative mutant of VEGF-D which dimerizes with native VEGF-D and blocks binding of the dimer to a VEGF receptor. The mutagenic techniques described herein can be used to map which determinants of VEGF-D protein participate in the intermolecular interactions involved in, for example, binding of VEGF-D protein to a VEGF receptor (e.g., VEGFR-2, VEGFR-3).

VEGF-D protein variants that do not bind a VEGF receptor can be generated through various techniques known in the art. For example, VEGF-D protein variants can be made by mutagenesis, such as by introducing discrete point mutation(s), by insertion or deletion. Whether a change in the amino acid sequence of a peptide results in a VEGF-D protein variant lacking one or more functional activities of a native VEGF-D protein can be readily determined by testing the variant for a native VEGF-D protein functional activity. For example, a brain tissue sample expressing a VEGF receptor can be contacted with a VEGF-D protein variant that lacks the ability to bind the receptor. The brain tissue sample can then be analyzed for receptor activation as well as angiogenesis. Absence of receptor activation would indicate the variant did not bind the receptor in the same manner as native VEGF-D protein.

Similarly, a VEGF receptor fragment, e.g. ligand binding domain, could be used in the methods of the invention to interfere with VEGF-D binding to endogenous, or native VEGF receptor. Over-expression of the extracellular, ligand binding domain of VEGFR-2 or VEGFR-3 would sequester VEGF-D away from native VEGF receptor (e.g., VEGFR-2, VEGFR-3). VEGF receptor variants can be generated through various techniques known in the art. To test if VEGF receptor variants are capable of binding VEGF-D, an immunoprecipitation analysis can be performed according to standard methods, for example, as described in Ausubel et al., supra. Antibodies that specifically recognize and bind either a VEGF-D protein or VEGF receptor variant protein are useful in methods of the invention.

Inhibiting Angiogenesis

The invention provides a method for inhibiting angiogenesis associated with brain cancer in a mammal. In one embodiment, a molecule that interferes with VEGF-D binding to a VEGF-D receptor is administered to a brain cancer of the mammal in an amount effective to inhibit angiogenesis. According to the methods of the invention, the molecule can specifically bind VEGFR-2 as well as VEGFR-3. In another embodiment, the molecule can bind VEGF-D. A molecule that specifically binds VEGF-D can be an antibody that specifically binds VEGF-D.

Administration of Compositions

The compositions described above may be administered to animals including human beings in any suitable formulation. For example, anti-angiogenic molecules may be formulated in pharmaceutically acceptable carriers or diluents such as physiological saline or a buffered salt solution. Suitable carriers and diluents can be selected on the basis of mode and route of administration and standard pharmaceutical practice. A description of exemplary pharmaceutically acceptable carriers and diluents, as well as pharmaceutical formulations, can be found in Remington's Pharmaceutical Sciences, a standard text in this field, and in USP/NF. Other substances may be added to the compositions to stabilize and/or preserve the compositions.

The compositions of the invention may be administered to animals by any conventional technique. The compositions may be administered directly to a target site by, for example, surgical delivery to an internal or external target site, or by catheter to a site accessible by a blood vessel. Other methods of delivery , e.g., liposomal delivery or diffusion from a device impregnated with the composition, are known in the art. The compositions may be administered in a single bolus, multiple injections, or by continuous infusion (e.g., intravenously). For parenteral administration, the compositions are preferably formulated in a sterilized pyrogen-free form.

Systemic (i.v.) with local interstitial drug delivery may be used according to the invention. The concept of convection enhanced delivery is becoming more attractive as an effective route of drug delivery into the brain. Laske et al., Nature Medicine 3, 1362-1368 (1997). Consequently, local delivery is the preferred approach to be evaluated clinically, since it may achieve high concentrations directly within the tumor mass and its vicinity.

Generally, compositions used in methods of the invention are introduced into a tumor cell using in vivo transduction techniques. Particularly, for in vivo delivery, the compositions will be formulated into pharmaceutical compositions and generally administered by direct injection into a tumor mass, injected intravenously into blood veins feeding the tumor mass, or administered into a tumor bed subsequent to tumor resection.

The compositions used in the invention may be precisely delivered into tumor sites, e.g., into gliomas or other intracranial tumors, by using stereotactic microinjection techniques. For example, the mammalian subject to be treated can be placed within a stereotactic frame base that is MRI-compatible and then imaged using high resolution MRI to determine the three-dimensional positioning of the particular tumor being treated. According to this technique, the MRI images are then transferred to a computer having the appropriate stereotactic software, and a number of images are used to determine a target site and trajectory for anti-angiogenic composition microinjection. Using such software, the trajectory is translated into three-dimensional coordinates appropriate for the stereotactic frame. For intracranial delivery, the skull will be exposed, burr holes will be drilled above the entry site, and the stereotactic apparatus positioned with the needle implanted at a predetermined depth. Tumor resection operations may be carried out prior to positioning of the stereotactic apparatus, if desired. A pharmaceutical composition containing an anti-angiogenic agent according to the invention can then be microinjected at the selected target site(s).

Effective Doses

The compositions described above are preferably administered to a mammal in an effective amount, that is, an amount capable of producing a desirable result in a treated subject (e.g., inhibiting angiogenesis and treating malignant tumors in the subject). Such a therapeutically effective amount can be determined as described below.

Toxicity and therapeutic efficacy of the compositions utilized in methods of the invention can be determined by standard pharmaceutical procedures, using either cells in culture or experimental animals to determine the LD_{50} (the dose lethal to 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Those compositions

that exhibit large therapeutic indices are preferred. While those that exhibit toxic side effects may be used, care should be taken to design a delivery system that minimizes the potential damage of such side effects. The dosage of preferred compositions lies preferably within a range that includes an ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized.

As is well known in the medical and veterinary arts, dosage for any one animal depends on many factors, including the subject's size, body surface area, age, the particular composition to be administered, time and route of administration, general health, and other drugs being administered concurrently. It is expected that an appropriate dosage for intratumoral administration of the compositions would be in the range of about 0.001 to 100 mg/kg body weight.

EXAMPLES

The present invention is further illustrated by the following specific examples. The examples are provided for illustration only and are not to be construed as limiting the scope or content of the invention in any way.

Example 1 – Materials and Methods

Cell lines and tissues: Glioblastoma multiforme cells lines A-172 MG, U-251 MG, DBTRG-50 MG, U-87 MG, U-373 MG, human GBM explant cells, G48a, CSML0 and CSML100 mouse breast carcinoma cells were grown in appropriate media. The CSML0 and CSML100 mouse breast cancer cells and GBM A172 MG glioblastoma cells were grown in Dulbecco's Modified Eagle's Medium (D-MEM) with 10% Fetal Calf Serum (FCS) (Life Technologies, Rockville, MD). U-251 MG cells were grown in D-MEM, 10% FCS, 0.1 mM MEM Non-Essential Amino Acids (NEAA) (Life Technologies), and 50 μg/ml Gentamicin Sulfate. GBM cell lines U-87 MG and U-373 MG were grown in Earle's Minimum Essential Medium (MEM), 10% FCS, 0.1 mM NEAA, 2 mM Glutamine (Life Technologies), and 100μg/ml Sodium Pyruvate. GMB cell line DBTRG-50 MG and human explant cells were grown in RPMI-1640 (Life Technologies) 10% FCS, 100 μg/ml Sodium Pyruvate, 100 μg/ml L-Cystine (Life Technologies), 20 μg/ml L-Proline (Sigma), 1x HT Supplement, consisting of .1 μM Sodium Hypoxanthine and 0.016 μM Thymidine, 5 units/ml Pennicilin G and 5 units/ml Streptomycin sulfate (Penn/Strep) (Life

Technologies). Normal Human Astrocytes (NHA) were grown in Astrocyte Growth Medium BulletKit^{R} (BioWhittaker). Normal HUV-EC-C were grown in F-12 Kaighn's medium (Life Technologies) with 10% FCS, 100 μg/ml Heparin (Sigma) and 30 μg/ml Endothelial Cell Growth Supplement (ECGS) (Sigma).

A retroviral vector was used to generate plasmid pMVfra-1. To produce replication-defective retroviruses, the GP+E packaging cell line was employed, which was maintained in appropriate media. Successfully transfected GP+E cells were selected in the presence of 800 μg/ml G418. Supernatants of virus-producing cell lines were used to infect CSML0 cells. Infected cells were selected in the presence of 400 μg/ml G418.

GBM tumors and non-malignant brain tissue, the latter obtained usually from the therapeutic resections for the treatment of epilepsy, were obtained from the operating room and snap frozen immediately, as described previously. Debinski et al. (1999) Clin. Cancer Res. 5: 985-990. Ten-micron sections of GBM were thaw-mounted onto chrom-alum slides. Slides were stored at –80° C until assayed. Sections were allowed to thaw and subsequently fixed for 10 min in acetone at -20° C.

Immunostaining: GBM cells lines, human explant cells (G48a), Human Umbilical Vein Endothelial Cells (HUV-EC-C) from ATCC (Rockville, MD), and normal human astrocytes (NHA) from BioWhittaker (Walkersville, MD) were grown overnight on sterile glass slides in the appropriate media. Slides were washed twice in PBS and fixed for 2 min in acetone at –80° C. Slides were washed twice in PBS and either used immediately or air-dried and stored at –80° C until assayed. In stimulation experiments, 10^4 SNB-19 GBM cells were plated on glass chamber slides and allowed to attach overnight. The cells were washed with PBS and serum-free media was applied. After 24 hr epidermal growth factor (EGF) or leukemia inhibitory factor (LIF) were added to cells at 5 and 20 ng/ml, respectively. The cells were processed for immunocytochemistry after 24 hr of stimulation period. Mouse monoclonal anti-VEGF-D (VD1) antibody was used. See, Achen et al. (2000) Eur. J. Biochem. 267: 2505-2515. It was employed at a final dilution of 1:500 (7.5 μg/ml). Other primary antibodies including rabbit polyclonal Fra-1 (1:100), c-Fos (1:100), c-Jun (1:150), and mouse monoclonal JunB (1:75) were purchased from

Santa Cruz Biotechnology (Santa Cruz, CA); and mouse monoclonal Factor VIII (1:150) and rabbit GFAP (1:500) were purchased from DAKO Chemical (Carpinteria, CA).

Slides were washed in two changes of PBS and blocked for 30 min with 10% (v/v) normal goat serum (NGS) in PBS at room temperature. Primary antibody was diluted in 1.5% NGS/PBS and incubated at room temperature for either 1 hr (VEGF-D, Factor VIII, and GFAP) or 2 hr (Fra-1, JunB, c-Fos, and c-Jun). Slides were washed in three changes of PBS for 10 min each. Secondary antibody, goat anti-rabbit Rhodamine (1:150), Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) or sheep anti-mouse Cy3 (1:250), Sigma (St. Louis, MO) was diluted in 1.5% NGS/PBS and incubated in the dark at room temperature for 45 min. For double-labeling experiments, the secondary antibodies were goat anti-mouse Oregon Green^R (1:200) (Molecular Probes, Oregon) and goat anti-rabbit Rhodamine (1:150). Slides were washed in 3 changes of PBS for 10 min each and mounted with Gel-Mount, Biomeda Corp. (Foster City, CA). Some slides were counterstained with Hoechst No. 33258 Nuclear Counterstain (DAPI).

Photomicrographs were taken at 40X magnification in all cases with a Hamamatsu C2400 digital camera. Background was normalized to the samples without primary antibody. Each set of images was taken exactly at the same exposure settings. Images were processed with Paint Shop Pro V 6.0 (Jasc software Inc., Eden Prairie, MN).

Western Blots: Cell lysates were prepared from sub-confluent cultures. Cells were washed twice in PBS and lysed in RIPA buffer (PBS, 1% Igepal CA-630; ICN Biomedicals, Inc. Costa Mesa, CA), 0.5% sodium deoxycholate (Fisher Scientific, Fair lawn, NJ), 0.1% SDS containing Mammalian Protease Inhibitor Cocktail (Sigma). GBM and non-malignant brain tumor samples were minced into small pieces while frozen and thawed in RIPA buffer with Mammalian Protease Inhibitor Cocktail. Lysates were passed through a 21-gauge needle to shear the DNA. 1 mM PMSF (Sigma) was added and the lysates were incubated on ice for 30-60 min. Non-solubilized debris was pelleted at 10,000 x g for 10 min. The supernatant was collected, aliquoted, and stored at –80° C until use. Normal human brain lysates were also purchased from Chemicon International, Inc. (Temecula, CA) and Clontech.

Lysates were run on either 12% or 15% SDS-PAGE. Proteins were transferred to PVDF membrane (Pierce, Rockford, IL) and blocked for 1 hr with 5% blotto (5% dry milk, PBS, 0.05% Tween-20). Membranes were incubated with primary antibody diluted in blotto for 40 min at room temperature while shaking. Antibodies included: anti-mouse VEGF-D antibody (40% cross-reactivity with human VEGF-D; 1:500) from R&D Systems, and Fra-1 (1:100) from Santa Cruz Research Antibodies. Following three five-minute washes in PBS/0.05% Tween-20, membranes were incubated in secondary antibody conjugated with horseradish peroxidase (goat anti-mouse IgG or goat anti-rabbit IgG) at a dilution of 1:10,000 or 1:15,000 in 5% blotto for 40 min at room temperature while shaking. Membranes were washed in several changes of PBS and detection was performed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce). Membranes were exposed to autoradiographic film X-OMAT AR for up to 5 min. Films were scanned in a transparency scanner at a pixel size of 88 X 88 micron (Molecular Dynamics, Sunnyvale, CA). The images were compiled in Paint Shop Pro V 6.0.

cDNA arrays: Atlas Oncogene/Tumor Suppressor Arrays were purchased from Clontech and 1 μg of poly(A)+ RNA was labeled with [α-^{33}P]dATP according to the manufacturer. Membranes were pre-hybridized overnight at 68° C in ExpressHyb (Clontech) containing 0.1 mg/ml sheared salmon sperm DNA. Labeled cDNA probe was denatured and added to the pre-hybridization solution and the membranes were hybridized overnight at 68° C. Membranes were then washed twice in 2X SSC/1% SDS for 20 min followed by two washes in 0.1% SSC/0.5% SDS at 68° C. The membranes were exposed to autoradiographic film for up to 10 days at –70° C. The arrays contain cDNA specific fragments for oncogenes, such as c-fos, junB, and c-myc. Housekeeping genes included ubiquitin, liver glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and phospholipase. RNA used for the cDNA micro-array assays was isolated from sub-confluent cultures of GBM cells using the acid-guanidium isothiocyanate-phenol-chloroform method. Chomczynski P, and Sacchi N (1987) Analyt. Biochem. 162: 156-159. Poly(A)+ RNA was further isolated using the Oligotex mRNA Kit (Qiagen Inc, Valencia, CA). Normal Human Brain Poly(A)+ RNA was purchased from Clontech (Clontech Laboratories, Inc., Palo Alto, CA).

Karyotyping: The karyotypes of HGA cells analyzed in this study were performed in a blinded fashion by clinical cytogeneticists at the Cancer Genetics Laboratory, Genetics & IVF Institute, Fairfax, VA.

Example 2 – Normal Brain Versus GBM Immunoreactivity for VEGF-D

To establish the expression of VEGF-D in normal brain, immunofluorescence studies were performed using sections of non-malignant brain tissue. Using ten randomly selected specimens of human brain, some immunoreactivity could be detected in normal brain using an anti-VEGF-D antibody VD1. Some tissue sections of non-malignant brain showed a weak staining for VEGF-D, which was localized primarily to the cell cytoplasm and cell processes. The remaining samples showed sporadic and even poorer staining, if at all. On the contiguous sections of the same piece of non-malignant brain, the staining of endothelial cell-associated von Willebrand Factor (Factor VIII) was evident, scattered and corresponded to the expected picture of vessel density and size seen in non-malignant brain. Small caliber vessels imprinted by the staining with the antibody against Factor VIII were seen in all sections. The background fluorescence in these experiments, i.e. without primary antibody added, was low.

Using exactly the same conditions as for immunofluorescence studies in non-malignant brain, a distinctively different picture of VEGF-D staining in malignant brain tissues has emerged. GBM sections demonstrated high levels of immunofluoresence for VEGF-D when compared with non-malignant brain and controls. VEGF-D showed a pattern of staining most compatible with cytoplasmic localization, i.e. diffused within the malignant astrocytic cells and also concentrated in their processes. All of the ten randomly selected tumors showed this pattern suggesting a remarkably high prevalence rate of VEGF-D over-production/ accumulation in GBMs.

GBMs are known to be highly vascular, which can be demonstrated by staining an antigen inherent to endothelial cells. Therefore, an antibody against Factor VIII was used in parallel immunofluorescence studies with tissue specimens; it was isotype-matched to the anti-VEGF-D antibody VD1. Anti-Factor VIII antibody immunofluorescence staining showed a characteristic pattern of hyperplastic vessels present on contiguous tissue sections of the same GBM as used for VEGF-D staining.

Factor VIII staining demonstrated larger and more complex blood vessels in GBM tissue than seen in non-malignant brain.

The pattern of VEGF-D staining in GBM was strongly suggestive of astrocytoma cells expressing this angiogenic factor. In order to document this directly, a double-staining experiment was performed using several GBM specimens. GBM sections were stained for glial-fibrillary acidic protein (GFAP), which is the most specific cytoplasmic marker for astrocytoma cells available. See, McKeever PE (1998) J. Histochem. Cytochem. 46: 585-594. GFAP staining of GBM revealed a typical cytoplasmic localization that outlines both astrocytoma cell bodies and their at times tube-like processes. VEGF-D staining co-localized with the staining for GFAP. GBM specimen #8 was stained with DAPI, a nuclear counter-stain, which further underlined the cytoplasmic localization of VEGF-D. Thus, a prominent presence of VEGF-D in the cytosol of astrocytoma cells of GBM was established *in situ*.

Example 3- Astrocytoma Cells Synthesize VEGF-D

The pattern of VEGF-D staining in GBM tissue specimens indicated that the tumor astrocytoma cells were the principal source of *in situ*-detected VEGF-D. In order to document that astrocytoma cells in fact express VEGF-D, several GBM cell lines were analyzed for both VEGF-D and Factor VIII immuno-reactivity. Six tumor cell lines studied showed a prominent immunofluorescent staining for VEGF-D. GBM cells, such as A-172 MG, G48a, U-87 MG, DBTRG-50 MG, U-251 MG, and U-373 MG demonstrated a diffuse cytoplasmic staining pattern. This diffuse cytoplasmic pattern provided a characteristic nuclear outline generated by VEGF-D. In contrast, the analysis of the non-malignant cell lines, human endothelial umbilical vein cells (HUV-EC-C) and normal human astrocytes (NHA), demonstrated low levels of immunoreactivity for VEGF-D, although this was more pronounced in NHA than in HUV-EC-C. Furthermore, HUV-EC-C stained prominently for Factor VIII in the cytoplasm and was the only cell line positive for this endothelial cell marker used in the experiment. Background fluorescent staining was low in all specimens and cell lines examined when using an irrelevant mouse IgG_1 or no primary antibody.

VEGF-D is an X-linked factor and therefore the karyotypes of astrocytoma cells used in experiments described herein were analyzed. Five out of six established cell lines exhibited abnormal ploidy for chromosome X, the most frequent alteration being an additional chromosome X.

The high content of VEGF-D immunoreactivity in GBM tumor specimens and GBM cell lines indicated VEGF-D protein therein. Cell lysates of four of the cell lines were analyzed by SDS-PAGE and Western blotting performed with the anti-VEGF-D antibody. A single protein band was detected with an approximate molecular weight of 48 kDa. This protein band corresponds to the full-length VEGF-D. Stacker et al. (1999) J. Biol. Chem. 274: 32127-32136. The GBM cell lines U-251 MG and DBTRG-50 MG possessed this protein, but it was not found in the lysates from either the astrocyte (NHA) or endothelial (HUV-EC-C) cell lines. These data, taken together with the specific immunostaining of tissues and cells, indicate that GBM tumor cells produce abundant amounts of VEGF-D, an X-linked angiogenic factor.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is: